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and diagnostic uses thereof, nucleic acid sequences encoding all or part of OSF, antibodies to and antagonists of OSF, and assays for OSF, and provides therapeutics for the prevention and treatment of diseases involving bone tissue including osteoporosis, Paget's disease, and osteopetrosis.

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(54) Title: CHIMERIC LEPTIN FUSED TO IMMUNOGLOBULIN DOMAIN AND USE

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(57) Abstract

Chimeric leptin which are proteins comprising leptin or a mutant or a variant thereof fused to a human immunogobulin domain. One favoured immunoglobulin domain is the human immunoglobulin Fc domain. The chimeric derivatives of leptin have, despite their large molecular size, good pharmacological activity combined with prolonged clearance rates. These derivatives of leptin are therefore indicated to be particularly useful for the treatment or prophylaxis of obesity or diseases and conditions associated with obesity such as atherosclerosis, hypertension and type II diabetes.

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(54) Title: HUMAN OBESITY GENE

(57) Abstract

The invention includes isolated DNA molecules that encode two different forms of the human *ob* gene product which regulate obesity in mammals, especially humans. Preferred embodiments of the native DNA encoding these two protein forms are disclosed. The invention further embraces vectors comprising the DNA, and methods for expressing these *ob* gene products.

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HUMAN OBESITY GENE

This Application is a Continuation-in-Part of Application Serial Number 08/384,493, filed February 6, 1995.

The invention belongs to the general field of molecular biology as applied to biopharmaceutical research and development. The invention includes DNA compounds, vectors and methods useful for expressing proteins that help regulate the body's volume of adipose tissue.

Obesity, especially upper body obesity, is a common and very serious public health problem in the United States and throughout the world. According to recent statistics, more than 25% of the U.S. population and 27% of the Canadian population are over weight. Kuczmarski, Amer. J. of Clin. Nut. 55: 495S - 502S (1992); Reeder *et al.*, Can. Med. Ass. J., 23: 226-233 (1992). Upper body obesity carries the highest risk factor known for Type II Diabetes and is a significant risk factor for cardiovascular disease and cancer as well. Recent cost estimates for medical complications associated with obesity are $150 billion world wide. The problem has now become so serious that the Surgeon General has begun a national initiative to combat obesity in America.

Hypertension, dyslipidemia, and insulin resistance are the primary pathologies associated with obesity. Many studies have demonstrated that weight reduction through diet and exercise dramatically improves these serious medical conditions. Unfortunately, obese individuals generally fail to significantly reduce their body mass through diet and exercise and have a near 95% failure rate. This failure may be due to genetically inherited factors that contribute to increased appetite, preference for high calorie foods, reduced physical activity, reduced lipolytic metabolism, and increased lipogenic metabolism. This indicates that people inheriting these genetic traits are prone to becoming obese regardless of their efforts to combat the condition.

Therefore, new pharmacological agents that can reverse obesity in spite of genetic predisposition are needed.

The *ob /ob* mouse model of obesity and diabetes is known to carry an autosomal recessive trait linked to a mutation in the sixth chromosome. Recently, Zhang and co-workers published the positional cloning of a mouse gene linked to this condition. Zhang *et al.* Nature 372: 425-32 (1994). This report discloses a mouse cDNA sequence encoding a 167 amino acid protein that is expressed exclusively in adipose tissue and compares this mouse *ob* gene product to a human homolog. The report also discloses a point mutation resulting in the conversion of an Arg codon to a stop codon at position 105. This mutant gene is postulated to expresses a truncated protein that lacks the biological function of the complete intact protein.

Physiologist have long postulated that excess fat cells laid down through overeating signals the brain that the body is obese which, in turn, causes the body to eat less and burn more fuel. G. R. Hervey, Nature 227: 629-631 (1969). Parabiotic experiments support a "feedback" model and suggest that a circulating peptide hormone may regulate the size of the body's fat depot. The newly disclosed *ob* gene product mentioned above is now believed to be such a hormone.

The present invention is based on the unexpected discovery of a new form of the obesity gene that was cloned from a human adipose tissue library. Therefore this invention is useful for producing what is currently believed to be biologically active anti-obesity proteins useful for treating obesity and reducing the risk for Type II diabetes, cardiovascular diseases, and cancer.

The invention is drawn to isolated nucleic acid molecules consisting of a nucleotide sequences that encode proteins having the amino acid sequences of SEQ ID NO: 2 and SEQ ID NO: 6. Recombinant DNA vectors and host cells

comprising such nucleic molecules make up further embodiments of the invention. Processes for producing anti-obesity proteins comprising culturing such host cells and isolating proteins comprising an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 6 are also claimed.

For purposes of the present invention, as disclosed and claimed herein, the following terms and abbreviations are defined as follows:

Base pair (bp) -- refers to DNA or RNA. The abbreviations A,C,G, and T correspond to the 5'-monophosphate forms of the nucleotides (deoxy)adenine, (deoxy)cytidine, (deoxy)guanine, and (deoxy)thymine, respectively, when they occur in DNA molecules. The abbreviations U,C,G, and T correspond to the 5'-monophosphate forms of the nucleosides uracil, cytidine, guanine, and thymine, respectively when they occur in RNA molecules. In double stranded DNA, base pair may refer to a partnership of A with T or C with G. In a DNA/RNA heteroduplex, base pair may refer to a partnership of T with U or C with G.

DNA -- Deoyxribonucleic acid.

RNA -- Ribonucleic acid

Nucleic acid molecule -- DNA or RNA.

Plasmid -- an extrachromosomal self-replicating genetic element.

Reading frame -- the nucleotide sequence from which translation occurs "read" in triplets by the translational apparatus of tRNA, ribosomes and associated factors, each triplet corresponding to a particular amino acid. Because each triplet is distinct and of the same length, the coding sequence must be a multiple of three. A base pair insertion or deletion (termed a frameshift mutation) may result in two different proteins being coded for by the same DNA segment. To insure against this, the triplet codons corresponding to the desired polypeptide must be aligned in multiples of three

from the initiation codon, i.e. the correct "reading frame" must be maintained. In the creation of fusion proteins containing a chelating peptide, the reading frame of the DNA sequence encoding the structural protein must be maintained in the DNA sequence encoding the chelating peptide.

Recombinant DNA Cloning Vector -- any autonomously replicating agent including, but not limited to, plasmids and phages, comprising a DNA molecule to which one or more additional DNA segments can or have been added.

Recombinant DNA Expression Vector -- any recombinant DNA cloning vector in which a promoter has been incorporated.

Recombinant DNA Vector -- A Recombinant DNA Expression Vector or a Recombinant DNA Cloning Vector

Replicon -- A DNA sequence that controls and allows for autonomous replication of a plasmid or other vector.

Transcription -- the process whereby information contained in a nucleotide sequence of DNA is transferred to a complementary RNA sequence.

Translation -- the process whereby the genetic information of messenger RNA is used to specify and direct the synthesis of a polypeptide chain.

Vector -- a replicon used for the transformation of cells in gene manipulation bearing polynucleotide sequences corresponding to appropriate protein molecules which, when combined with appropriate control sequences, confer specific properties on the host cell to be transformed. Plasmids, viruses, and bacteriophage are suitable vectors, since they are replicons in their own right. Artificial vectors are constructed by cutting and joining DNA molecules from different sources using restriction enzymes and ligases. Vectors include Recombinant DNA cloning vectors and Recombinant DNA expression vectors.

The amino acids abbreviations are accepted by the United States Patent and Trademark Office as set forth in 37 C.F.R. § 1.822 (b)(2) (1993). Unless otherwise indicated the amino acids are in the L configuration.

In one embodiment, the invention provides DNA molecules that encode a novel form of the human *ob* gene product which is defined by the following amino acid sequence.

SEQ ID NO: 2

```
1               5                   10                  15
Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys

                20                  25                  30
Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Ala Gln Ser Val

                35                  40                  45
Ser Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly Leu

                50                  55                  60
His Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val

                65                  70                  75
Tyr Gln Gln Ile Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln

                80                  85                  90
Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu

                95                  100                 105
Ala Phe Ser Lys Ser Cys His Leu Pro Trp Ala Ser Gly Leu Glu

                110                 115                 120
Thr Leu Asp Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr Ser

                125                 130                 135
Thr Glu Val Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp

                140                 145
Met Leu Trp Gln Leu Asp Leu Ser Pro Gly Cys-COOH
```

A preferred coding region for the above embodiment is defined by the following DNA sequence.

SEQ ID NO: 1

5'- GTA CCT ATC CAG AAA GTC CAG GAT GAC ACC AAA ACC CTC ATC
AAG ACA ATT GTC ACC AGG ATC AAT GAC ATT TCA CAC GCG CAG TCA
GTC TCC TCC AAA CAG AAA GTC ACC GGT TTG GAC TTC ATT CCT GGG
CTC CAC CCC ATC CTG ACC TTA TCC AAG ATG GAC CAG ACA CTG GCA
GTC TAC CAA CAG ATC CTC ACC AGT ATG CCT TCC AGA AAC GTG ATC
CAA ATA TCC AAC GAC CTG GAG AAC CTC CGG GAT CTT CTT CAC GTG
CTG GCC TTC TCT AAG AGC TGC CAC TTG CCC TGG GCC AGT GGC CTG
GAG ACC TTG GAC AGC CTG GGG GGT GTC CTG GAA GCT TCA GGC TAC
TCC ACA GAG GTG GTG GCC CTG AGC AGG CTG CAG GGG TCT CTG CAG
GAC ATG CTT TGG CAG CTG GAC CTG TCT CCC GGG TGC - 3'

A more preferred coding region for the above embodiment is defined by the following DNA sequence which is the naturally occurring cDNA.

SEQ ID NO: 3

5'- GTG CCC ATC CAA AAA GTC CAA GAT GAC ACC AAA ACC CTC ATC
AAG ACA ATT GTC ACC AGG ATC AAT GAC ATT TCA CAC GCG CAG TCA
GTC TCC TCC AAA CAG AAA GTC ACC GGT TTG GAC TTC ATT CCT GGG
CTC CAC CCC ATC CTG ACC TTA TCC AAG ATG GAC CAG ACA CTG GCA
GTC TAC CAA CAG ATC CTC ACC AGT ATG CCT TCC AGA AAC GTG ATC
CAA ATA TCC AAC GAC CTG GAG AAC CTC CGG GAT CTT CTT CAC GTG
CTG GCC TTC TCT AAG AGC TGC CAC TTG CCC TGG GCC AGT GGC CTG
GAG ACC TTG GAC AGC CTG GGG GGT GTC CTG GAA GCT TCA GGC TAC
TCC ACA GAG GTG GTG GCC CTG AGC AGG CTG CAG GGG TCT CTG CAG
GAC ATG CTG TGG CAG CTG GAC CTC AGC CCT GGG TGC - 3'

A further embodiment of the invention provides DNA molecules that encode a second novel form of the human *ob* gene product having a Gln-28 deletion. This embodiment is defined by the following amino acid sequence.

SEQ ID NO: 6

1	5	10	15
Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile Lys			
20	25	30	
Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Ser Val Ser			
35	40	45	
Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly Leu His			
50	55	60	
Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val Tyr			
65	70	75	
Gln Gln Ile Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln Ile			
80	85	90	
Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala			
95	100	105	
Phe Ser Lys Ser Cys His Leu Pro Trp Ala Ser Gly Leu Glu Thr			
110	115	120	
Leu Asp Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr			

125 130 135
Glu Val Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Met

140 145
Leu Trp Gln Leu Asp Leu Ser Pro Gly Cys-COOH

A preferred coding sequence for this shorter form of the *ob* gene product is defined by the following DNA sequence.

SEQ ID NO: 5

5'- GTA CCT ATC CAG AAA GTC CAG GAT GAC ACC AAA ACC CTC ATC
AAG ACA ATT GTC ACC AGG ATC AAT GAC ATT TCA CAC ACG TCA GTC
TCC TCC AAA CAG AAA GTC ACC GGT TTG GAC TTC ATT CCT GGG CTC
CAC CCC ATC CTG ACC TTA TCC AAG ATG GAC CAG ACA CTG GCA GTC
TAC CAA CAG ATC CTC ACC AGT ATG CCT TCC AGA AAC GTG ATC CAA
ATA TCC AAC GAC CTG GAG AAC CTC CGG GAT CTT CTT CAC GTG CTG
GCC TTC TCT AAG AGC TGC CAC TTG CCC TGG GCC AGT GGC CTG GAG
ACC TTG GAC AGC CTG GGG GGT GTC CTG GAA GCT TCA GGC TAC TCC
ACA GAG GTG GTG GCC CTG AGC AGG CTG CAG GGG TCT CTG CAG GAC
ATG TTG TGG CAG CTG GAC CTG TCT CCC GGG TGC - 3'

A more preferred coding sequence for this shorter form of the *ob* gene product is defined by the following naturally occurring cDNA sequence.

SEQ ID NO: 7

5'- GTG CCC ATC CAA AAA GTC CAA GAT GAC ACC AAA ACC CTC ATC
AAG ACA ATT GTC ACC AGG ATC AAT GAC ATT TCA CAC ACG TCA GTC
TCC TCC AAA CAG AAA GTC ACC GGT TTG GAC TTC ATT CCT GGG CTC
CAC CCC ATC CTG ACC TTA TCC AAG ATG GAC CAG ACA CTG GCA GTC
TAC CAA CAG ATC CTC ACC AGT ATG CCT TCC AGA AAC GTG ATC CAA
ATA TCC AAC GAC CTG GAG AAC CTC CGG GAT CTT CTT CAC GTG CTG
GCC TTC TCT AAG AGC TGC CAC TTG CCC TGG GCC AGT GGC CTG GAG

ACC TTG GAC AGC CTG GGG GGT GTC CTG GAA GCT TCA GGC TAC TCC
ACA GAG GTG GTG GCC CTG AGC AGG CTG CAG GGG TCT CTG CAG GAC
ATG CTG TGG CAG CTG GAC CTC AGC CCT GGG TGC - 3'

The claimed DNA sequences are useful for expressing the *ob* gene product either by direct expression or as fusion protein. When the claimed sequences are used in a fusion gene, the resulting product will require enzymatic or chemical cleavage. A variety of peptidases which cleave a polypeptide at specific sites or digest the peptides from the amino or carboxy termini (e.g. diaminopeptidase) of the peptide chain are known. Furthermore, particular chemicals (e.g. cyanogen bromide) will cleave a polypeptide chain at specific sites. The skilled artisan will appreciate the modifications necessary to the amino acid sequence (and synthetic or semi-synthetic coding sequence if recombinant means are employed) to incorporate site-specific internal cleavage sites. See U.S. Patent No. 5,126,249; Carter P., Site Specific Proteolysis of Fusion Proteins, Ch. 13 in Protein Purification: From Molecular Mechanisms to Large Scale Processes, American Chemical Soc., Washington, D.C. (1990).

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required.

To effect the translation of the desired protein, one inserts the engineered synthetic DNA sequence in any of a plethora of appropriate recombinant DNA expression vectors through the use of appropriate restriction endonucleases. The claimed protein is a relatively large protein. A synthetic coding sequence is designed to possess restriction endonuclease cleavage sites at either end of the transcript

to facilitate isolation from and integration into these expression and amplification and expression plasmids. The isolated cDNA coding sequence may be readily modified by the use of synthetic linkers to facilitate the incorporation of this sequence into the desired cloning vectors by techniques well known in the art. The particular endonucleases employed will be dictated by the restriction endonuclease cleavage pattern of the parent expression vector to be employed. The restriction sites are chosen so as to properly orient the coding sequence with control sequences to achieve proper in-frame reading and expression of the claimed protein.

In general, plasmid vectors containing promoters and control sequences which are derived from species compatible with the host cell are used with these hosts. The vector ordinarily carries a replication origin as well as marker sequences which are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species (Bolivar, et al., Gene 2: 95 (1977)). Plasmid pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain or be modified to contain promoters and other control elements commonly used in recombinant DNA technology.

The desired coding sequence is inserted into an expression vector in the proper orientation to be transcribed from a promoter and ribosome binding site, both of which should be functional in the host cell in which the protein is to be expressed. An example of such an expression vector is a plasmid described in Belagaje et al., U.S. patent No. 5,304,493, the teachings of which are herein incorporated by reference. The gene encoding A-C-B proinsulin described in

U.S. patent No. 5,304,493 can be removed from the plasmid pRB182 with restriction enzymes NdeI and BamHI. The claimed DNA sequences of the present invention can be inserted into the plasmid backbone on a NdeI/BamHI restriction fragment cassette.

In general, procaryotes are used for cloning of DNA sequences in constructing the vectors useful in the invention. For example, E. coli K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include E. coli B and E. coli X1776 (ATCC No. 31537). These examples are illustrative rather than limiting.

Procaryotes also are used for expression. The aforementioned strains, as well as E. coli W3110 (prototrophic, ATCC No. 27325), bacilli such as Bacillus subtilis, and other enterobacteriaceae such as Salmonella typhimurium or Serratia marcescans, and various pseudomonas species may be used. Promoters suitable for use with prokaryotic hosts include the b-lactamase (vector pGX2907 [ATCC 39344] contains the replicon and b-lactamase gene) and lactose promoter systems (Chang et al., Nature, 275:615 (1978); and Goeddel et al., Nature 281:544 (1979)), alkaline phosphatase, the tryptophan (trp) promoter system (vector pATH1 [ATCC 37695] is designed to facilitate expression of an open reading frame as a trpE fusion protein under control of the trp promoter) and hybrid promoters such as the tac promoter (isolatable from plasmid pDR540 ATCC-37282). However, other functional bacterial promoters, whose nucleotide sequences are generally known, enable one of skill in the art to ligate them to DNA encoding the protein using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno sequence operably linked to the DNA encoding protein.

The claimed DNA molecules may also be recombinantly produced in eukaryotic expression systems. Preferred promoters controlling transcription in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. b-actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication. Fiers, et al., Nature, 273:113 (1978). The entire SV40 genome may be obtained from plasmid pBRSV, ATCC 45019. The immediate early promoter of the human cytomegalovirus may be obtained from plasmid pCMBr (ATCC 77177). Of course, promoters from the host cell or related species also are useful herein.

Transcription of the claimed DNA by higher eucaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively oriented and positioned independently and have been found 5' (Laimins, L. et al., PNAS 78:993 (1981)) and 3' (Lusky, M. L., et al., Mol. Cell Bio. 3:1108 (1983)) to the transcription unit, within an intron (Banerji, J. L. et al., Cell 33:729 (1983)) as well as within the coding sequence itself (Osborne, T. F., et al., Mol. Cell Bio. 4:1293 (1984)). Many enhancer sequences are now known from mammalian genes (globin, RSV, SV40, EMC, elastase, albumin, a-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 late enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding protein. The 3' untranslated regions also include transcription termination sites.

Expression vectors may contain a selection gene, also termed a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR, which may be derived from the BglII/HindIII restriction fragment of pJOD-10 [ATCC 68815]), thymidine kinase (herpes simplex virus thymidine kinase is contained on the BamHI fragment of vP-5 clone [ATCC 2028]) or neomycin (G418) resistance genes (obtainable from pNN414 yeast artificial chromosome vector [ATCC 37682]). When such selectable markers are successfully transferred into a mammalian host cell, the transfected mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow without a supplemented media. Two examples are: CHO DHFR$^-$ cells (ATCC CRL-9096) and mouse LTK$^-$ cells (L-M(TK-) ATCC CCL-2.3). These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual

cells which were not transformed with the DHFR or TK gene will not be capable of survival in nonsupplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, Southern P. and Berg, P., J. Molec. Appl. Genet. 1: 327 (1982), mycophenolic acid, Mulligan, R. C. and Berg, P. Science 209:1422 (1980), or hygromycin, Sugden, B. et al., Mol Cell. Biol. 5:410-413 (1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively.

A preferred vector for eucaryotic expression is pRc/CMV. pRc/CMV is commercially available from Invitrogen Corporation, 3985 Sorrento Valley Blvd., San Diego, CA 92121. To confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E. coli K12 strain DH10B (ATCC 31446) and successful transformants selected by antibiotic resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction and/or sequence by the method of Messing, et al., Nucleic Acids Res. 9:309 (1981).

Host cells may be transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled

artisan. The techniques of transforming cells with the aforementioned vectors are well known in the art and may be found in such general references as Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), or Current Protocols in Molecular Biology (1989) and supplements.

Preferred suitable host cells for expressing the vectors encoding the claimed proteins in higher eucaryotes include: African green monkey kidney line cell line transformed by SV40 (COS-7, ATCC CRL-1651); transformed human primary embryonal kidney cell line 293,(Graham, F. L. et al., J. Gen Virol. 36:59-72 (1977), Virology 77:319-329, Virology 86:10-21); baby hamster kidney cells (BHK-21(C-13), ATCC CCL-10, Virology 16:147 (1962)); chinese hamster ovary cells CHO-DHFR$^-$ (ATCC CRL-9096), mouse Sertoli cells (TM4, ATCC CRL-1715, Biol. Reprod. 23:243-250 (1980)); african green monkey kidney cells (VERO 76, ATCC CRL-1587); human cervical epitheloid carcinoma cells (HeLa, ATCC CCL-2); canine kidney cells (MDCK, ATCC CCL-34); buffalo rat liver cells (BRL 3A, ATCC CRL-1442); human diploid lung cells (WI-38, ATCC CCL-75); human hepatocellular carcinoma cells (Hep G2, ATCC HB-8065);and mouse mammary tumor cells (MMT 060562, ATCC CCL51).

In addition to prokaryotes, unicellular eukaryotes such as yeast cultures may also be used. Saccharomyces cerevisiae, or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (ATCC-40053, Stinchcomb, et al., Nature 282:39 (1979); Kingsman et al., Gene 7:141 (1979); Tschemper et al., Gene 10:157 (1980)) is commonly used. This plasmid already contains the trp gene which provides a selection marker for a mutant strain of yeast lacking the

ability to grow in tryptophan, for example ATCC no. 44076 or PEP4-1 (Jones, Genetics 85:12 (1977)).

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (found on plasmid pAP12BD ATCC 53231 and described in U.S. Patent No. 4,935,350, June 19, 1990) or other glycolytic enzymes such as enolase (found on plasmid pAC1 ATCC 39532), glyceraldehyde-3-phosphate dehydrogenase (derived from plasmid pHcGAPC1 ATCC 57090, 57091), zymomonas mobilis (United States Patent No. 5,000,000 issued March 19, 1991), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which contain inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein (contained on plasmid vector pCL28XhoLHBPV ATCC 39475, United States Patent No. 4,840,896), glyceraldehyde 3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose (GAL1 found on plasmid pRY121 ATCC 37658) utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., European Patent Publication No. 73,657A. Yeast enhancers such as the UAS Gal from Saccharomyces cerevisiae (found in conjunction with the CYC1 promoter on plasmid YEpsec--hIlbeta ATCC 67024), also are advantageously used with yeast promoters.

The following examples will help describe how the invention is practiced and will illustrate the characteristics of the claimed DNA molecules, vectors, host cells, and methods of the invention.

EXAMPLE 1

PCR Amplification

Degenerate primers were designed based on the published amino acid sequence of the human *ob* gene. The primers were prepared for use in polymerase chain reaction (PCR) amplification methods using a Model 380A DNA synthesizers (PE-Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404). Forward primers OB.F1M (5-GG GG CAT ATG AGG GTA CCT ATC CAG AAA GTC CAG GAT GAC AC)(SEQ ID NO: 9) and OB.F2H (5-GG GG CAT ATG AGG GTA CCC ATC CAG AAG GTG CAG GAC GA)(SEQ ID NO: 10) and reverse primers OB.R1M (5-GG GG GGATC GAT AAT TTA GCA TCC AGG GCT AAG ATC CAA CTG CCA AAG CAT)(SEQ ID NO: 11) and OB.R2H (5-GG GG GGATC CTA TTA GCA CCC GGG AGA CAG GTC CAG CTG CCA CAA CAT)(SEQ ID NO: 12) were mixed together with a PCR-ready human fat cell cDNA as the template (Clontech Laboratories, Inc., 4030 Fabian Way, Palo Alto, CA 94303; Item #7128-1).

The 2 sets of PCR amplifications were performed using 2.5 units of Amplitag DNA polymerase (Perkin Elmer Cetus) or 2 units of Vent DNA polymerase (New England Biolabs) in 100 uL reactions. PCR reactions contained 1 uL of human fat cell cDNA, 10 pmol of each primer (all four were mixed). The following conditions were used for "Touchdown PCR": 2 cycles: 94°Cx30 sec, 60°Cx30 sec, 72°Cx45 sec 2 cycles: 94°Cx30 sec, 56°Cx30 sec, 72°Cx45 sec; 2cycles: 94°Cx30 sec, 52°Cx30 sec, 72°Cx45 sec; 2cycles: 94°Cx30 sec, 48°Cx30 sec, 72°Cx45 sec; 2 cycles: 94°Cx30 sec, 44°Cx30 sec, 72°Cx45 sec: 28 cycles 94°Cx30 sec, 52°Cx30 sec, 72°Cx45 sec

The resultant PCR reactions products were run on a 1% agarose gel and a band of an approximate 450 bp in size was visualized by ethidium bromide staining. This band was present in both sets of PCR reactions. The bands were excised and reamplified using above conditions in 30 cycles (94x30 sec, 52x30, 72x45). The PCR product obtained using

Vent DNA polymerase was gel purified and cloned into a pCR-SCRIPT cloning vector (Stratagene). The vector was then used to transform E. coli cells. Plasmid DNA was isolated from 20 white colonies of E. coli and samples from three clones were sequenced. Two such colonies, E. coli DH10B/pOJ717 containing SEQ ID NO: 1 and E. coli DH10B/pOJ718 containing SEQ ID NO: 5, were deposited with the Northern Regional Research Laboratories (NRRL) under terms of the Budapest Treaty and are available under Accession Numbers B-21408 and B-21409 respectively.

Example 2

Vector Construction

A plasmid containing the DNA sequence encoding the desired claimed protein is constructed to include NdeI and BamHI restriction sites. The plasmid carrying the cloned PCR product is digested with NdeI and BamHI restriction enzymes. The small ~ 450bp fragment is gel-purified and ligated into the vector pRB182 from which the coding sequence for A-C-B proinsulin is deleted. The ligation products are transformed into E. coli DH10B (commercially available from GIBCO-BRL) and colonies growing on tryptone-yeast (DIFCO) plates supplemented with 10 mg/mL of tetracycline are analyzed. Plasmid DNA is isolated, digested with NdeI and BamHI and the resulting fragments are separated by agarose gel electrophoresis. Plasmids containing the expected ~ 450bp NdeI to BamHI fragment are kept. E. coli K12 RV308 (available from the NRRL under deposit number B-15624) are transformed with this second plasmid, resulting in a culture suitable for expressing the protein.

The techniques of transforming cells with the aforementioned vectors are well known in the art and may be found in such general references as Maniatis, et al. (1988) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New

York or Current Protocols in Molecular Biology (1989) and supplements. The techniques involved in the transformation of E. coli cells used in the preferred practice of the invention as exemplified herein are well known in the art. The precise conditions under which the transformed E. coli cells are cultured is dependent on the nature of the E. coli host cell line and the expression or cloning vectors employed. For example, vectors which incorporate thermoinducible promoter-operator regions, such as the c1857 thermoinducible lambda-phage promoter-operator region, require a temperature shift from about 30 to about 40 degrees C. in the culture conditions so as to induce protein synthesis.

In the preferred embodiment of the invention, E. coli K12 RV308 cells are employed as host cells but numerous other cell lines are available such as, but not limited to, E. coli K12 L201, L687, L693, L507, L640, L641, L695, L814 (E. coli B). The transformed host cells are then plated on appropriate media under the selective pressure of the antibiotic corresponding to the resistance gene present on the expression plasmid. The cultures are then incubated for a time and temperature appropriate to the host cell line employed.

Proteins which are expressed in high-level bacterial expression systems characteristically aggregate in granules or inclusion bodies which contain high levels of the overexpressed protein. Kreuger et al., in Protein Folding, Gierasch and King, eds., pgs 136-142 (1990), American Association for the Advancement of Science Publication No. 89-18S, Washington, D.C. Such protein aggregates must be solubilized to provide further purification and isolation of the desired protein product. Id. A variety of techniques using strongly denaturing solutions such as guanidinium-HCl and/or weakly denaturing solutions such as dithiothreitol

(DTT) are used to solubilize the proteins. Gradual removal of the denaturing agents (often by dialysis) in a solution allows the denatured protein to assume its native conformation. The particular conditions for denaturation and folding are determined by the particular protein expression system and/or the protein in question.

Preferably, the present DNA sequences are expressed with a dipeptide leader sequence encoding Met-Arg or Met-Tyr as described in U.S. Patent No. 5,126,249, herein incorporated by reference. This approach facilitates the efficient expression of proteins and enables rapid conversion to the active protein form with Cathepsin C or other dipeptidylpeptidases. The purification of proteins is by techniques known in the art and includes reverse phase chromatography, affinity chromatography, and size exclusion.

EXAMPLE 3

Biological Testing

Parabiotic experiments suggest that a protein is released by peripheral adipose tissue and that the protein is able to control body weight gain in normal, as well as obese mice. Therefore, the most closely related biological test is to inject the test article by any of several routes of administration (i.v., s.c., i.m., i.p., or by minipump or cannula) and then to monitor food and water consumption, body weight gain, plasma chemistry or hormones (glucose, insulin, ACTH, corticosterone, GH, T4) over various time periods.

Suitable test animals include normal mice (ICR, etc.) and obese mice (*ob/ob* , Avy/a, KK-Ay, tubby, fat). The *ob/ob* mouse model of obesity and diabetes is generally accepted in the art as being indicative of the obesity condition. Controls for non-specific effects for these injections are done using vehicle with or without the active agent of similar composition in the same animal monitoring

the same parameters or the active agent itself in animals that are thought to lack the receptor (db/db mice, fa/fa or cp/cp rats). Proteins demonstrating activity in these models will demonstrate similar activity in other mammals, particularly humans.

Since the target tissue is expected to be the hypothalamus where food intake and lipogenic state are regulated, a similar model is to inject the test article directly into the brain (e.g. i.c.v. injection via lateral or third ventricles, or directly into specific hypothalamic nuclei (e.g. arcuate, paraventricular, perifornical nuclei). The same parameters as above could be measured, or the release of neurotransmitters that are known to regulate feeding or metabolism could be monitored (e.g. NPY, galanin, norepinephrine, dopamine, b-endorphin release).

Similar studies are accomplished *in vitro* using isolated hypothalamic tissue in a perifusion or tissue bath system. In this situation, the release of neurotransmitters or electrophysiological changes is monitored.

Proteins expressed by the claimed DNA sequences are believed to be active in at least one of the above biological tests and are anti-obesity agents. As such, the claimed DNA sequences are useful for preparing protein compositions for treating obesity and disorders implicated by obesity. However, the claimed DNA sequences are also useful for preparing immunogens to raise antibodies for diagnostic use.

Claims

1. An isolated nucleic acid molecule consisting of a nucleotide sequence that encodes a protein having the following amino acid sequence:

NH_2-Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile
Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Ala Gln Ser
Val Ser Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly
Leu His Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala
Val Tyr Gln Gln Ile Leu Thr Ser Met Pro Ser Arg Asn Val Ile
Gln Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Val
Leu Ala Phe Ser Lys Ser Cys His Leu Pro Trp Ala Ser Gly Leu
Glu Thr Leu Asp Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr
Ser Thr Glu Val Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln
Asp Met Leu Trp Gln Leu Asp Leu Ser Pro Gly Cys-COOH
(SEQ ID NO: 2).

2. A nucleic acid molecule of **Claim 1** consisting of DNA having the following nucleotide sequence:

5'- GTA CCT ATC CAG AAA GTC CAG GAT GAC ACC AAA ACC CTC ATC
AAG ACA ATT GTC ACC AGG ATC AAT GAC ATT TCA CAC GCG CAG TCA
GTC TCC TCC AAA CAG AAA GTC ACC GGT TTG GAC TTC ATT CCT GGG
CTC CAC CCC ATC CTG ACC TTA TCC AAG ATG GAC CAG ACA CTG GCA
GTC TAC CAA CAG ATC CTC ACC AGT ATG CCT TCC AGA AAC GTG ATC
CAA ATA TCC AAC GAC CTG GAG AAC CTC CGG GAT CTT CTT CAC GTG
CTG GCC TTC TCT AAG AGC TGC CAC TTG CCC TGG GCC AGT GGC CTG
GAG ACC TTG GAC AGC CTG GGG GGT GTC CTG GAA GCT TCA GGC TAC
TCC ACA GAG GTG GTG GCC CTG AGC AGG CTG CAG GGG TCT CTG CAG
GAC ATG CTT TGG CAG CTG GAC CTG TCT CCC GGG TGC - 3'
(SEQ ID NO: 1)

3. A nucleic acid molecule of **Claim 1** consisting of DNA having the following nucleotide sequence:

5'- GTG CCC ATC CAA AAA GTC CAA GAT GAC ACC AAA ACC CTC ATC
AAG ACA ATT GTC ACC AGG ATC AAT GAC ATT TCA CAC GCG CAG TCA
GTC TCC TCC AAA CAG AAA GTC ACC GGT TTG GAC TTC ATT CCT GGG
CTC CAC CCC ATC CTG ACC TTA TCC AAG ATG GAC CAG ACA CTG GCA
GTC TAC CAA CAG ATC CTC ACC AGT ATG CCT TCC AGA AAC GTG ATC
CAA ATA TCC AAC GAC CTG GAG AAC CTC CGG GAT CTT CTT CAC GTG
CTG GCC TTC TCT AAG AGC TGC CAC TTG CCC TGG GCC AGT GGC CTG

GAG ACC TTG GAC AGC CTG GGG GGT GTC CTG GAA GCT TCA GGC TAC
TCC ACA GAG GTG GTG GCC CTG AGC AGG CTG CAG GGG TCT CTG CAG
GAC ATG CTG TGG CAG CTG GAC CTC AGC CCT GGG TGC - 3'
(SEQ ID NO: 3)

4. An isolated nucleic acid molecule consisting of a nucleotide sequence that encodes a protein having the following amino acid sequence:

NH_2-Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys Thr Leu Ile
Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr Ser Val
Ser Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro Gly Leu
His Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala Val
Tyr Gln Gln Ile Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln
Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu
Ala Phe Ser Lys Ser Cys His Leu Pro Trp Ala Ser Gly Leu Glu
Thr Leu Asp Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr Ser
Thr Glu Val Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp
Met Leu Trp Gln Leu Asp Leu Ser Pro Gly Cys-COOH
(SEQ ID NO: 6)

5. A nucleic acid molecule of **Claim 4** consisting of DNA having the following nucleotide sequence:

5'- GTA CCT ATC CAG AAA GTC CAG GAT GAC ACC AAA ACC CTC ATC
AAG ACA ATT GTC ACC AGG ATC AAT GAC ATT TCA CAC ACG TCA GTC
TCC TCC AAA CAG AAA GTC ACC GGT TTG GAC TTC ATT CCT GGG CTC
CAC CCC ATC CTG ACC TTA TCC AAG ATG GAC CAG ACA CTG GCA GTC
TAC CAA CAG ATC CTC ACC AGT ATG CCT TCC AGA AAC GTG ATC CAA
ATA TCC AAC GAC CTG GAG AAC CTC CGG GAT CTT CTT CAC GTG CTG
GCC TTC TCT AAG AGC TGC CAC TTG CCC TGG GCC AGT GGC CTG GAG
ACC TTG GAC AGC CTG GGG GGT GTC CTG GAA GCT TCA GGC TAC TCC
ACA GAG GTG GTG GCC CTG AGC AGG CTG CAG GGG TCT CTG CAG GAC
ATG TTG TGG CAG CTG GAC CTG TCT CCC GGG TGC - 3'
(SEQ ID NO: 5)

6. A nucleic acid molecule of **Claim 4** consisting of DNA having the following nucleotide sequence:

5'- GTG CCC ATC CAA AAA GTC CAA GAT GAC ACC AAA ACC CTC ATC
AAG ACA ATT GTC ACC AGG ATC AAT GAC ATT TCA CAC ACG TCA GTC
TCC TCC AAA CAG AAA GTC ACC GGT TTG GAC TTC ATT CCT GGG CTC
CAC CCC ATC CTG ACC TTA TCC AAG ATG GAC CAG ACA CTG GCA GTC
TAC CAA CAG ATC CTC ACC AGT ATG CCT TCC AGA AAC GTG ATC CAA
ATA TCC AAC GAC CTG GAG AAC CTC CGG GAT CTT CTT CAC GTG CTG
GCC TTC TCT AAG AGC TGC CAC TTG CCC TGG GCC AGT GGC CTG GAG
ACC TTG GAC AGC CTG GGG GGT GTC CTG GAA GCT TCA GGC TAC TCC

ACA GAG GTG GTG GCC CTG AGC AGG CTG CAG GGG TCT CTG CAG GAC ATG CTG TGG CAG CTG GAC CTC AGC CCT GGG TGC - 3'
(SEQ ID NO: 7)

7. A recombinant DNA vector comprising a nucleic acid molecule of **Claim 1**.

8. A recombinant DNA vector comprising the DNA molecule of **Claim 2**.

9. A recombinant DNA vector comprising the DNA molecule of **Claim 3**.

10. A recombinant DNA vector comprising a nucleic acid molecule of **Claim 4**.

11. A recombinant DNA vector comprising the DNA molecule of **Claim 5**.

12. A recombinant DNA vector comprising the DNA molecule of **Claim 6**.

13. A recombinant host cell comprising a vector of **Claim 7**.

14. A recombinant host cell comprising a vector of **Claim 8**.

15. A recombinant host cell comprising a vector of **Claim 9**.

16. A recombinant host cell comprising a vector of **Claim 10**.

17. A recombinant host cell comprising a vector of **Claim 11**.

18. A recombinant host cell comprising a vector of **Claim 12**.

19. A recombinant host cell of **Claim 8** that is E. coli DH10B/pOJ717 on deposit with the NRRL under Accession Number B-21408.

20. A recombinant host cell of **Claim 11** that is E. coli DH10B/pOJ718 on deposit with the NRRL under Accession Number B-21409.

21. A process for producing an anti-obesity protein comprising;

a) culturing a host cell of **Claim 13**; and,

b) isolating a protein comprising an amino acid sequence of SEQ ID NO: 2.

22. A process for producing an anti-obesity protein comprising;

a) culturing a host cell of **Claim 14**; and,

b) isolating a protein comprising an amino acid sequence of SEQ ID NO: 6.

23. A process for producing an anti-obesity protein comprising;

a) culturing the host cell of **Claim 19**; and,

b) isolating a protein comprising an amino acid sequence of SEQ ID NO: 2.

24. A process for producing an anti-obesity protein comprising;

a) culturing a host cell of **Claim 20**; and,

b) isolating a protein comprising an amino acid sequence of SEQ ID NO: 6.